REMARKS

The present invention relates in part to methods for staining cells for detection by flow cytometry. In particular, the present claims relate to methods for catalyzing the deposition of tyramide in an analyte-specific manner for the detection of intracellular analytes. The methods of the instant claims can provide at least a 10-fold greater signal than that obtained by standard flow cytometric techniques.

Claims 1-61 are currently pending in the application, with claims 1-33 under consideration by the Examiner. Claims 34-61, which were removed from consideration in a restriction requirement, are cancelled herein. Claims 1, 2, 5, 18, and 32 are amended herein. These amendments do not introduce new matter or require a new search, and are merely provided to aid the Examiner in understanding the instantly claimed invention, as discussed hereinafter. The amendments to the claims are not made for purposes of patentability, and do not further limit the claims as originally filed. Support for methods in which a signal is obtained that is at least 10-fold greater than a signal obtainable by standard flow cytometry methods using isotype/subtype matched nonspecific immunoglobulin as a negative control may be found in the specification, *e.g.*, on page 10, lines 12-21.

Notwithstanding the foregoing, Applicant expressly reserves the right to pursue subject matter no longer claimed in the instant application in one or more applications which may claim priority hereto. Applicant respectfully requests reconsideration of the claimed invention in view of the foregoing amendments and the following remarks.

Non-Art Related Remarks

35 U.S.C. § 112, second paragraph

Applicant respectfully traverses in part the rejection of claims 1-33 under 35 U.S.C. § 112, second paragraph, alleging that the claims are indefinite for failing to particularly point out and distinctly claim the present invention.

When determining definiteness, the proper standard to be applied is "whether one skilled in the art would understand the bounds of the claim when read in the light of the specification." *Credle v. Bond*, 30 USPQ2d 1911, 1919 (Fed.Cir.1994). See also *Miles Laboratories, Inc. v. Shandon, Inc.*, 27 USPQ2d 1123, 1127 (Fed.Cir.1993) ("If the claims read in the light of the specification reasonably apprise those skilled in the art of the scope of the invention, § 112 demands no more.").

Applicant respectfully submits that the foregoing amendments to the claims render the rejection of claims 1, 2, 5, 18, and 32 moot.

Applicant respectfully traverses the rejection of claims 3-32 as allegedly lacking proper antecedent basis in reciting "A method according to claim...". The language to which the Examiner objects indicates that each claim is dependent, and refers back to and further limits a previous claim, in accordance with 37 C.F.R. § 1.75. Each of the rejected claims are either multiply dependent claims, or depend from multiply dependent claims. MPEP 608.01(n) specifically describes the use of the indefinite article in the context of multiple dependent claims.

Furthermore, there is nothing of record to indicate that the skilled artisan would understand such language in the context of multiple dependent claims, but somehow would not reasonably understand the very same language in a singular dependent claim. Applicant respectfully requests that the Examiner cite support for her belief that the use of an indefinite article in dependent claims is not allowed, or explain why the skilled artisan is not reasonably apprised of the scope of the invention by the claims as written.

Applicant respectfully submits that the claims meet the standard of 35 U.S.C. §112, second paragraph, and request that the rejection be reconsidered and withdrawn.

Art-Related Remarks

35 U.S.C. §§102

Applicant respectfully traverses the rejection of claims 1, 2, 5, 10, 14-18, 25-26, and

28-33 under 35 U.S.C. § 102(b) as allegedly being anticipated by Karkmann *et al.*, *J. Immunol. Meth.* 230: 113-120, 1999; and claims 1, 2, 5, 11-19, and 23-33 as allegedly being anticipated by Lollini, *Immunological Blackboard* Vol. 1.

To anticipate a claim, the reference must teach every element of the claim. "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). "The identical invention must be shown in as complete detail as is contained in the ... claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989). *See also, In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991); MPEP § 2143.

Applicant respectfully submits that the instant claims distinguish over the Karkmann *et al.* and Lollini publications, in that the cited publications do not disclose any methods in which cells comprising an intracellular analyte of interest are specifically labeled, or in which the signal obtained is at least 10-fold greater than a signal obtainable by standard flow cytometry methods using isotype/subtype matched nonspecific immunoglobulin as a negative control, as required by the instant claims. Applicant notes that the claims have been amended herein to clarify that the fold enhancement of signal provided by the instant claims is in comparison to a signal obtainable using isotype/subtype matched nonspecific immunoglobulin controls.

To assist the Examiner in interpreting the cited publications, Applicant submits herewith a declaration of Dr. David Kaplan. In the declaration, Dr. Kaplan describes why the skilled artisan would not understand the cited publications to disclose the claimed invention. For example, neither publication uses the proper controls required for the skilled artisan to acknowledge that specific staining is obtained, nor that the signal obtained is at least 10-fold greater than a signal obtainable by standard flow cytometry methods. Moreover, the conditions disclosed in the cited publications for tyramide staining of intracellular analytes do not provide specific staining or a 10-fold enhancement of signal in comparison to standard flow cytometry methods when isotype/subtype matched nonspecific immunoglobulin is used as a negative

control, due to nonspecific background obtained when using the disclosed conditions. *See, e.g.*, Kaplan Declaration, paragraphs 6 and 8.

Because the cited publications fail to disclose each and every element of the instant claims, no *prima facie* case of anticipation has been established. Applicants, therefore, respectfully request that the rejections under 35 U.S.C. §102 be reconsidered and withdrawn.

35 U.S.C. § 103

Applicant respectfully traverses the rejection of claims 3-4, 6-9, and 20-22 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Karkmann *et al.* or Lollini.

To establish a *prima facie* case of obviousness, three criteria must be met: there must be some motivation or suggestion, either in the cited references or in knowledge available to the ordinarily skilled artisan, to modify or combine the references; there must be a reasonable expectation of success in combining the references; and the references must teach or suggest all of the claim limitations. *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991) *See also,* MPEP §2143.

As discussed in detail above and in the Kaplan Declaration accompanying this paper, the skilled artisan would not understand the cited publications to disclose the claimed invention, as the conditions disclosed in the cited publications for tyramide staining of intracellular analytes do not provide specific staining or a 10-fold enhancement of signal in comparison to standard flow cytometry methods when isotype/subtype matched nonspecific immunoglobulin is used as a negative control. Nothing of record provides any working methods for obtaining the claimed invention, or any indication as to which of the myriad variables in an assay method should be considered in order to obtain the claimed invention.

The suggestion of a general approach, when the prior art gives only general guidance as to the particular form of the claimed invention or how to achieve it, is not sufficient to support a *prima facie* case of obviousness. *See*, MPEP § 2145(X)(B). Without the express teachings of the instant specification, the artisan would be forced to simply try every possible combination of

variables, hoping to find a useful method. Because the skilled artisan would not have a reasonable expectation of success in modifying the publications to obtain the instantly claimed methods, Applicant respectfully submits that no *prima facie* case of anticipation has been established.

Moreover, even if the Examiner is correct that a *prima facie* case of obviousness has been established, such a *prima facie* case may be rebutted by evidence of superior results. *See, e.g.*, MPEP §2144.09. As noted in the Kaplan Declaration, a comparison of the conditions described in the present application to those disclosed in the cited publications indicates that, while the present application can provide at least a 10-fold enhancement of an antigen-specific signal, and as shown in Figure 4, as much as a 150-fold enhancement of signal, the cited publications do not. See, e.g., Kaplan Declaration, paragraphs 9 and 10. Any increase in signal that may be obtained is considered critical to those of skill in the art, as an increase in specific signal indicates an increase in the detection sensitivity of the flow cytometric assay. Because these methods are often used in diagnosis of human diseases, this increase in detection sensitivity can translate directly into improved diagnosis. Applicant respectfully submits that the evidence of superior results provided herewith and in the instant specification rebuts any *prima facie* case of obviousness that has been established.

With regard to claims 6-9 and 20-22, these claims refer to the use of high percentages (at least 50%) of serum in a medium used during performance of the claimed methods. While the Examiner states that "[i]t has long been settled to be no more than routine experimentation for one of ordinary skill in the art to discover an optimum value of a result effective variable" (Paper No. 10, page 7), the cited art must first recognize that the variable is "result effective." MPEP § 2144.05(II)(B). Nothing of record indicates that the addition of high percentages of serum in a tyramide deposition method is a "result effective variable." In fact, nothing of record suggests that serum should be used at any concentration when labeling an intracellular analyte by deposition of tyramide. Instead, the cited publications indicate that only extremely low

percentages (0.5% and 1%) of bovine serum albumin are appropriate in a tyramide deposition method.

The only possible "result effective variables" disclosed in the Karkmann *et al.* publication are the type of antibody-enzyme conjugate, the cell density, and the type of fluorescent molecule used during labeling. Karkmann *et al.*, page 115, right column, through 116, left column. The Lollini publication does not disclose any variables as being "result effective." Because the addition of high percentages of serum when labeling an intracellular analyte by deposition of tyramide was not recognized to be a "result effective variable," the " routine experimentation" argument presented by the Examiner cannot support a *prima facie* case of obviousness. *See, e.g.*, *In re Yates*, 211 USPQ 1149 (CCPA 1981) (failure to provide objective evidence supporting an allegation that a variable is "result effective," fails to establish a prima facie case of obviousness based upon "routine experimentation").

Therefore, because no *prima facie* case of obviousness has been established, or, in the alternative, that any *prima facie* case of obviousness may have been established has been rebutted, Applicants respectfully request that the rejection under 35 U.S.C. §103 be reconsidered and withdrawn.

CONCLUSION

Applicants respectfully submit that the pending claims are in condition for allowance. An early notice to that effect is earnestly solicited. Should any matters remain outstanding, the Examiner is encouraged to contact the undersigned at the address and telephone number listed below so that they may be resolved without the need for additional action and response thereto.

Respectfully submitted,

Date: October 16, 2002

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Appendix A: Marked up version of claims, indicating amendments

1. (Amended) A method of detecting the presence of an intracellular analyte in one or more cells by flow cytometry, the method comprising:

a) fixing and permeabilizing said cells;

b) catalyzing the deposition of tyramide in said cells comprising said intracellular analyte;

c) contacting said cells with a detectable label that directly or indirectly binds to tyramide, whereby said cells comprising said intracellular analyte are specifically labeled; and

d) detecting a signal from said cells comprising said detectable label using a flow cytometric device, wherein said signal indicates the presence of said intracellular analyte, and [whereby] wherein said signal is at least 10-fold greater than a signal obtainable by standard flow cytometry methods using isotype/subtype matched nonspecific immunoglobulin as a negative control.

2. (Amended) A method of detecting the presence of an intracellular analyte in one or more cells by flow cytometry, the method comprising:

a) fixing and permeabilizing said cells;

b) catalyzing the deposition of tyramide conjugated to a detectable label in said cells comprising said intracellular analyte, whereby said cells comprising said intracellular analyte are specifically labeled; and

c) detecting a signal from said cells comprising said detectable label using a flow cytometric device, wherein said signal indicates the presence of said intracellular analyte, and [whereby] wherein said signal is at least 10-fold greater then a signal obtainable by standard flow

cytometry methods using isotype/subtype matched nonspecific immunoglobulin as a negative control.

3. (Reiterated) A method according to claim 1 or 2, wherein said signal is at least 20-fold greater then a signal obtainable by standard flow cytometry methods.

4. (Reiterated) A method according to claim 1 or 2, wherein said signal is at least 50-fold greater than a signal obtainable by standard flow cytometry methods.

5. (Amended) A method according to claim 1 or 2, wherein said catalyzing step comprises:

(i) incubating the fixed and permeabilized cells with a binding partner that specifically binds to said analyte, wherein said binding partner is conjugated to an enzyme [capable of catalyzing] that, in the presence of substrate for said enzyme and tyramide, catalyzes the deposition of tyramide in said cells comprising said intracellular analyte;

(ii) removing unbound binding partner from said cells; and

(iii) contacting bound binding partner with tyramide and said enzyme substrate, whereby said enzyme catalyzes the deposition of tyramide in said cells comprising said intracellular analyte.

6. (Reiterated) A method according to claim 5, wherein said binding partner is incubated with said fixed and permeabilized cells in a medium comprising at least about 50% serum.

7. (Reiterated) A method according to claim 6, wherein said serum is fetal bovine serum.

8. (Reiterated) A method according to claim 7, wherein said medium comprises at least about 95% fetal bovine serum.

9. (Reiterated) A method according to claim 8, wherein said medium further comprises about 0.2% saponin.

10. (Reiterated) A method according to claim 1 or 2, wherein said cells are permeabilized in a medium comprising saponin.

11. (Reiterated) A method according to claim 1 or 2, wherein said cells are permeabilized in a medium comprising methanol.

12. (Reiterated) A method according to claim 5, wherein said bound binding partner is contacted with tyramide in a medium comprising an aprotic solvent.

13. (Reiterated) The medium of claim 12, wherein said medium comprises at least about 5% of an aprotic solvent selected from the group consisting of acetone, dimethyl sulfoxide, acetonitrile, and dimethyl formamide.

14. (Reiterated) A method according to claim 1 or 2, wherein said detectable label is a fluorochrome.

15. (Reiterated) A method according to claim 14, wherein said fluorochrome comprises a fluorescent molecule selected from the group consisting of fluorescein, phycoerythrin, CY5, allophycocyanine, Texas Red, Peridenin chlorophyll, and cyanine.

16. (Reiterated) A method according to claim 5, wherein said enzyme is selected from the group consisting of hydrolysases, peroxidase, oxidase, esterases, glycosidases and phosphatases.

17. (Reiterated) A method according to claim 5, wherein said enzyme is horseradish peroxidase.

18. (Amended) A method according to claim 1 or 2, wherein said catalyzing step comprises:

(i) incubating the fixed and permeabilized cells with a first binding partner that specifically binds to said first binding partner, wherein said second binding partner comprises an enzyme, wherein said second binding partner is conjugated to an enzyme [capable of catalyzing]

that, in the presence of substrate for said enzyme and tyramide, catalyzes the deposition of tyramide in said cells comprising said intracellular analyte;

(ii) removing unbound second binding partner from said cells; and

(iii) contacting bound second binding partner with tyramide and said enzyme substrate, whereby said enzyme catalyzes the deposition of tyramide in said cells comprising said intracellular analyte.

19. (Reiterated) A method according to claim 18, wherein said second binding partner is an immunoglobulin-enzyme conjugate.

20. (Reiterated) A method according to claim 19, wherein said second binding partner is incubated with said fixed and permeabilized cells in a medium comprising at least about 50% serum.

21. (Reiterated) A method according to claim 20, wherein said serum is fetal bovine serum.

22. (Reiterated) A method according to claim 12, wherein said medium comprises at least about 95% fetal bovine serum.

23. (Reiterated) A method according to claim 19, wherein said immunoglobulin-peroxidase, immunoglobulin-hydrolase, immunoglobulin-oxidase, immunoglobulin-gycosidase and immunoglobulin-phosphatase.

24. (Reiterated) A method according to claim 23, wherein said immunoglobulin-enzyme conjugate is immunoglobulin-horseradish peroxidase.

25. (Reiterated) A method according to claim 1 or 2, wherein said one or more cells are one or more mammalian cells.

26. (Reiterated) A method according to claim 25, wherein said one or more mammalian cells are selected form the group consisting of basal cells, epithelial cells, erythrocytes, platelets,

lymphocytes, T-cells, B-cells, natural killer cells, granulocytes, monocytes, mast cells, Jurkat cells, neurocytes, neuroblasts, cytomegalic cells, dendritic cells, macrophages, blastomeres, endothelial cells, HeLa cells, tumor cells, interstitial cells, Kupffer cells, Langerhans' cells, Langhans cells, littoral cells, tissue cells, adipose cells, CHO cells, KFL9, and K562 cells.

27. (Reiterated) A method according to claim 1 or 2, wherein said one or more cells are cultured cells.

28. (Reiterated) A method according to claim 1 or 2, wherein said intracellular analyte is selected from the group consisting of intracellular cytokines, antigens, viral antigens, nuclear antigens, cytoplasmic antigens, organellar antigens, enzymes, ctyoskeletal molecules, glycolipids, lipids, glycans, chaperones, RNA, DNA, messenger RNA, ribosomal RNA, signal transduction proteins, and structural proteins.

29. (Reiterated) A method according to claim 1 or 2, wherein said intracellular analyte is not a natural component of said one or more cells.

30. (Reiterated) A method according to claim 1 or 2, wherein said intracellular analyte cannot be detected by standard flow cytometry methods.

31. (Reiterated) A method according to claim 1 or 2, wherein said one or more cells are obtained from a patient.

32. (Amended) A method according to claim 31, wherein [said signal] <u>the presence of said intracellular analyte</u> is correlated to a diagnosis of a disease in said patient.

33. (Reiterated) A kit for performing a method according to claims 1 or 2.